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DOCUMENT-IDENTIFIER: US 20040241655 A1
TITLE: Conditional touchdown multiplex polymerase chain reaction

Abstract Paragraph:
A high-throughput and cost-effective method for simultaneous amplification of target DNA sequences with high fidelity and workable rate is achieved by a two-stage amplification incorporating multiplex PCR with conditional touchdown strategies. This improved multiplex PCR comprises a simultaneous PCR and a specific PCR, and either one or both of the amplification steps are performed with a touchdown strategy, of which loose touchdown strategy is applied with a temperature lower than the optimized annealing temperature, and stringent touchdown strategy is applied with a temperature higher than the optimized annealing temperature.

Summary of Invention Paragraph:
[0012] Multiplex PCR

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[0013] PCR multiplexing, the simultaneous amplification of two or more loci in a single PCR reaction, see Chamberlain, "Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification", Nucleic Acids Res (1988) 16:11141-56, is a powerful technique that considerably reduces the time and cost, as well as required genomic DNA samples, for genetic analysis. This method has been successfully applied to many areas of DNA testing, including determination of genetic polymorphisms; however, the pooling of a plurality of PCR primers in a single reaction could cause many problems, including increased formation of spurious PCR products and primer dimmers, and biased amplification of shorter DNA fragments. All these potential problems, if applied to SNP genotyping, lead to incorrect results. A detailed description about various conditions and encountered difficulties of multiplex PCR has been discussed by Henegariu et al., "Multiplex PCR: critical parameters and step-by-step protocol", BioTechniques (1997) 23:504-511.

Summary of Invention Paragraph:
[0017] Multiplex PCR pooling multiple PCR primer pairs in a same reaction could cause many problems, including increased formation of spurious PCR products and primer dimmers, and enhanced amplification of shorter DNA fragments, and thus compromises its workable rate.

Summary of Invention Paragraph:
[0018] Due to the potential problems of multiplex PCR described above, the successful rate hardly reaches more than 50% from documentation as the technique was employed in SNP genotyping.

Summary of Invention Paragraph:
[0019] Several prior arts have been proposed to solve various problems in this field. For example, in U.S. Pat. No. 5,736,365, Walker et al. use multiplex Strand Displacement Amplification (SDA) in a single amplification reaction which is capable of simultaneously identifying M. tuberculosis and providing a screen for substantially all of the clinically relevant species of Mycobacteria. U.S. Pat. Nos. 5,882,856 and 6,207,372 issued to Shuber provide universal primer sequence for

multiplex DNA amplification to allow multiplex PCR reactions to be designed and carried out without elaborate optimization steps, irrespective of the potentially divergent properties of the different primers used and to simultaneously produce equivalent amounts of each one of many amplification products. Diamandis et al. propose method, reagents and kit for diagnosis and targeted screening for p53 mutations, in U.S. Pat. No. 6,071,726, for rapid and cost effective diagnosis of p53 mutations in a sample of patients. These proposed methods did not deal with the above problems.

Summary of Invention Paragraph:
[0020] In U.S. Pat. Publication No. 20020058281, Matsuzaki et al. teach methods and compositions for multiplex amplification of nucleic acids, which permit the amplification of different sequences with the same efficiency so that approximately equimolar products result. This method needs high concentration of primers and uses single annealing temperature, and its PCR products are non-specific and its workable rate is very low. To solve the difficulty of using low amount of genomic DNA as template and higher number of pooled primer pairs in multiplex PCR, by use of hot start Taq polymerase in multiplexing amplification reactions, Nakamura et al. disclose a method for SNP typing in U.S. Pat. Publication No. 20020182622, which can genotype hundreds of thousands of SNP sites using a remarkably small amount of genomic DNA. However, this method still uses high concentration of primers and single annealing temperature, and by which the PCR products are non-specific and the workable rate is still low (not over 50%).

Summary of Invention Paragraph:
[0022] An object of the present invention is to provide a speedy and affordable method for simultaneous amplification of multiple target DNA sequences by multiplex PCR accompanying with conditional touchdown strategies. The invented method can be applied, but not limited, to SNP genotyping. Furthermore, according to the present invention, the required amount of DNA template applied to SNP genotyping is dramatically reduced compared with conventional genotyping methods.

Summary of Invention Paragraph:
[0023] The incorporation of touchdown PCR to multiplex PCR in the invented method is intended to solve the difficulty of misprimed PCR products encountered in PCR multiplexing. The touchdown PCR is a PCR variant that has been adopted to circumvent more complicated optimization processes for determining annealing temperature. It involves decreasing the annealing temperature by 1.degree. C. every second cycle to a `touchdown` annealing temperature, which is then used for 10 or so cycles. The spirit is that any differences in temperature Tm between correct and incorrect annealing gives a 2-fold difference in product amount per cycle, thus enriching for the correct product over any incorrect products, see Don et al., "Touchdown PCR to circumvent spurious priming during gene amplification", Nucleic Acids Res (1991) 19:4008. The invented method that incorporates touchdown strategies to multiplex PCR is highly valuable when large number of primer pairs is required, especially in the case of SNP genotyping.

Summary of Invention Paragraph:
[0025] In a conditional touchdown multiplex PCR, according to the present invention, a simultaneous PCR and a specific PCR are comprised. In the simultaneous PCR, the amplification is performed to increase the primers annealing to templates, and the specific PCR is to enrich the abundance of the designated sequence. Either one or both of the annealing temperatures for the simultaneous PCR and specific PCR employ a touchdown strategy. Particularly, the annealing temperature for the specific PCR is higher than that for the simultaneous PCR.

Summary of Invention Paragraph:
[0026] In a preferred embodiment of the present invention, a genotyping method comprises a simultaneous amplification step for the multiplex PCR with loose touchdown strategy (LTS), and a specific amplification step to the PCR products